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# Dehydration-regulated processing of late embryogenesis abundant protein in a desiccation-tolerant nematode

Kshamata Goyal[a,1], Claudia Pinelli[b], Sarah L. Maslen[c], Rakesh K. Rastogi[d], Elaine Stephens[c], Alan Tunnacliffe[a,*]

[a] *Institute of Biotechnology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QT, UK*
[b] *Department of Life Sciences, Second University of Naples, Via Vivaldi 43, 81100 Caserta, Italy*
[c] *Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge CB2 1EW, UK*
[d] *Department of Structural and Functional Biology, University of Naples Federico II, Via Cinthia, 80100 Napoli, Italy*

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**Abstract Late embryogenesis abundant (LEA) proteins occur in desiccation-tolerant organisms, including the nematode *Aphelenchus avenae*, and are thought to protect other proteins from aggregation. Surprisingly, expression of the LEA protein AavLEA1 in *A. avenae* is partially discordant with that of its gene: protein is present in hydrated animals despite low cognate mRNA levels. Moreover, on desiccation, when its gene is upregulated, AavLEA1 is specifically cleaved to discrete, smaller polypeptides. A processing activity was found in protein extracts of dehydrated, but not hydrated, nematodes, and main cleavage sites were mapped to 11-mer repeated motifs in the AavLEA1 sequence. Processed polypeptides retain function as protein anti-aggregants and we hypothesise that the expression pattern and cleavage of LEA protein allow rapid, maximal availability of active molecules to the dehydrating animal.**


*Keywords:* Anhydrobiosis; Desiccation stress; Desiccation tolerance; Late embryogenesis abundant protein; Molecular shield

## 1. Introduction

Anhydrobiotic organisms can survive almost complete desiccation by entering a state of metabolic arrest which is reversible on rehydration [1]. One response to desiccation common to many, perhaps all, anhydrobiotes is the production of highly hydrophilic proteins. The best characterised of these are the late embryogenesis abundant (LEA) proteins which are linked to the acquisition of desiccation tolerance in orthodox seeds, pollen and anhydrobiotic plants [2, and references therein]. Several groups of LEA proteins have been defined on the basis of expression pattern and sequence; for example, Group 3 LEA proteins are characterised by a repeating, loosely conserved, 11-mer amino acid motif. In addition, Group 3 LEA proteins are highly hydrophilic and natively unfolded in solution; unusually, they show increased folding on drying, but become unstructured again on rehydration [3,4].

Recently, LEA and LEA-like proteins or their genes have also been found in micro-organisms and invertebrate animal species [5], including the anhydrobiotic nematode *Aphelenchus avenae*, where expression of a Group 3 LEA protein gene is induced by dehydration [6]. The widespread occurrence of LEA proteins and genes suggests that plants, animals and micro-organisms might use these proteins in similar ways to combat water stress. LEA proteins have a stabilising function (reviewed in [7]) and recently several groups have shown that LEA, and other hydrophilic, proteins can preserve enzyme activity after desiccation and rehydration [8–10]. A possible mechanism for the protection observed is the prevention of water stress-induced aggregation of sensitive proteins [11]. Hydrophilic proteins might also function as membrane stabilisers [12] and it has been proposed that folding of LEA proteins on membranes occurs in a manner similar to that of α-synuclein, which has a role in vesicle management [13].

These studies improve our understanding of how LEA proteins behave in vitro, but there is a need for corresponding investigations of their in vivo biology, particularly in desiccation-tolerant animals, where there are no data on regulation of LEA proteins. Therefore, we have performed experiments to determine the distribution and behaviour of the LEA protein AavLEA1 in both hydrated and dehydrated nematodes. We report the surprising finding that, during nematode anhydrobiosis, AavLEA1 expression is partially out of phase with that of its mRNA, and that the protein is processed to smaller polypeptides which nevertheless retain anti-aggregation activity.

## 2. Material and methods

### 2.1. Immunostaining

Adult and juvenile nematodes, including embryonated eggs, were ruptured by freeze-cracking, stained with immunopurified anti-AavLEA1 and detected with either biotin-conjugated goat anti-rabbit serum (1:200), followed by a streptavidin/diaminobenzidine treatment, or a goat anti-rabbit FITC (fluorescein isothiocyanate; 1:200) [14]. In some preparations, cell nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole). Negative controls were performed using primary antibody preabsorbed with AavLEA1 (5 μM). A positive control comprised incubation of freeze-cracked worms with FMRFamide-specific antibodies (Affinity) which stained neurons strongly. Microscopy was performed with a Leica DMRB optical microscope and a digital Nikon Coolpix camera or a laser scanning confocal microscope (Leica TCSNT).

*Corresponding author. Fax: +44 1223 334162.
*E-mail addresses:* at10004@biotech.cam.ac.uk, a.tunnacliffe@biotech.cam.ac.uk (A. Tunnacliffe).

[1] Present address: Department of Zoology, University of Cambridge, Downing Street, Cambridge CB2 3EJ, UK.

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*2.2. RNA analysis*

*A. avenae* was exposed to 90% RH for various times (saturated solution of $BaCl_2$) at 25 °C prior to storage at −80 °C; controls were untreated. RNA isolation, Northern hybridisation and quantitative PCR were as described [15].

*2.3. SDS–PAGE and Western blotting*

About 15 μg of total protein was loaded per lane of 11% SDS slab gel and run in Bio-Rad mini-Protean 3 electrophoresis cells, with 25–50 ng of recombinant LEA protein as positive control [4]. Anti-14-3-3 (rabbit polyclonal) and lysates of EGF-stimulated A431 cells were used according to supplier's instructions (Upstate).

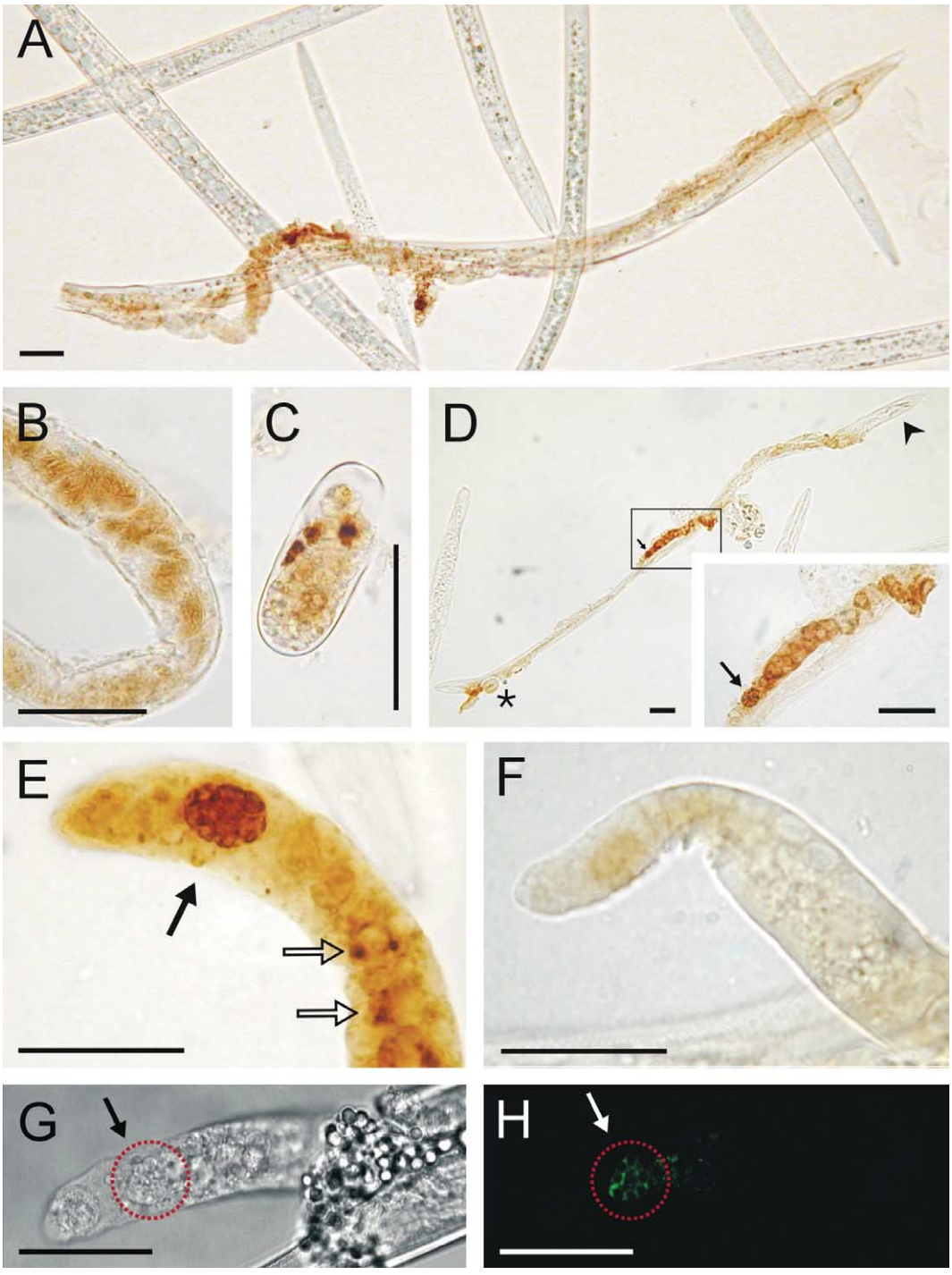


Fig. 1. Immunodetection of LEA protein in *A. avenae*. LEA protein immunoreaction (brown coloration) in (A) the body of a female worm (head at right); (B) embryonated eggs in the uterus; (C) an egg after laying; (D) a male worm (asterisk indicates head end, arrowhead posterior end). Gonadal immunoreactivity is strong as shown in the enlarged inset, where the arrow indicates probable germ cells. (E) A cluster of germ cells in the ovary showing strong LEA protein immunoreaction (black arrow); open arrows indicate immunoreactivity in the cytoplasm of growing oocytes. (F) Control experiment: lack of staining of ovary after preabsorbing antiserum with recombinant AavLEA1. Scale bar, A–F = 20 μm. (G) Nomarsky optics of extruded ovary, with strongly immunofluorescent stem cells in confocal image (H) circled and indicated with an arrow. Green fluorescence in the cytoplasm represents LEA protein. Scale bar, G, H = 16 μm.

*2.4. LEA protein processing and anti-aggregation assay*

After dialysis against distilled water, recombinant LEA protein (2 mg) was incubated with 10 μl (0.2 mg) of total nematode protein extract isolated from control and dried *A. avenae* in 100 μl reaction volume at ambient temperature for times indicated, with control incubations performed in water; 10 μl samples were analysed by SDS–PAGE. Gels were stained with Coommassie blue R-250 and apparent $M_r$ determined relative to standards (Sigma). The experiment was repeated several times. The desiccation stress aggregation assay was described previously [11].

*2.5. Mass spectrometry*

Intact masses for in vitro processed recombinant AavLEA1 were determined by MALDI-TOF-MS in linear mode directly after C4 ZipTip purification (Millipore). Intact and processed recombinant LEA proteins were digested with a few crystals of CNBr (Aldrich) dissolved in HPLC grade 70% formic acid in $H_2O$. The CNBr solution was added to the dried protein samples and incubated in the dark at room temperature for 16 h. The digestion mixture was dried down and analysed directly by MALDI-TOF-MS after C18 ZipTip (Millipore) purification. All mass spectra were recorded on a 4700 Proteomics analyzer with TOF/TOF optics (Applied Biosystems, Foster City, CA). This MALDI tandem mass spectrometer uses a 200 Hz frequency-tripled Nd:YAG laser operating at a wavelength of 355 nm. All linear MALDI-TOF spectra were calibrated externally using a standard protein calibration mixture containing insulin, thioredoxin and apomyoglobin (Applied Biosystems). Peptides derived from CNBr digestion of the processed recombinant protein were identified by nanoLC-MALDI-TOF/TOF tandem mass spectrometry. NanoLC was carried out as described [16], and the purified peptides were identified by MALDI-MS/MS. For MS/MS experiments, the collision energy was set at 1 kV and peptide ions were collided with air at a pressure of $7 \times 10^{-7}$ Torr.

## 3. Results and discussion

*3.1. LEA protein expression in nematode tissues*

If the LEA protein AavLEA1 has an important role in protecting *A. avenae* from desiccation damage, it should be expressed in many tissues of the animal. To test this, immunostaining experiments were performed using immunopurified polyclonal antisera raised against recombinant AavLEA1. Fig. 1A shows that AavLEA1 is present throughout the whole body length of female specimens, with the possible exception of the pharynx; it is also present in embryos *in utero* and after laying (Fig. 1B and C). Males are rare in our laboratory cultures, since reproduction is largely by meiotic parthenogenesis [17], but were also found to contain LEA protein in most tissues (Fig. 1D). An intriguing staining pattern was observed in both female and male gonads, which are readily extruded through breaks in the cuticle induced by freeze-cracking. A high level of expression of LEA protein was seen in a group of cells near, but not at, the distal tip of the gonad (Fig. 1D and E); these are probably germ line stem cells. The intensity of staining of other cells in the gonad was similar to that in the rest of the animal. When the antiserum was preincubated with recombinant AavLEA1, no significant staining was observed (Fig. 1F). Confocal microscopy of the heavily staining group of cells in the female gonad showed the presence of LEA protein in the cytoplasm, but not the nucleus (Fig. 1G and H), whose location was confirmed by counterstaining with DAPI (data not shown). These results demonstrate that AavLEA1 is present in all or most cells of both sexes of *A. avenae* and at all developmental stages, and that it is localised to the cytoplasm.

*3.2. Complex regulation of* A. avenae *LEA protein*

Previously, it was shown that expression of the gene, *Aav-lea-1*, encoding AavLEA1 is induced by dehydration of the nematode *A. avenae*, with very little mRNA being present in fully hydrated animals [6,15]. However, the data of Fig. 1 demonstrate the presence of LEA protein in nematodes harvested from apparently unstressed cultures. This was unexpected, since the amount of LEA protein in the animal should reflect that of its mRNA, assuming a simple relationship between transcription and translation. To investigate

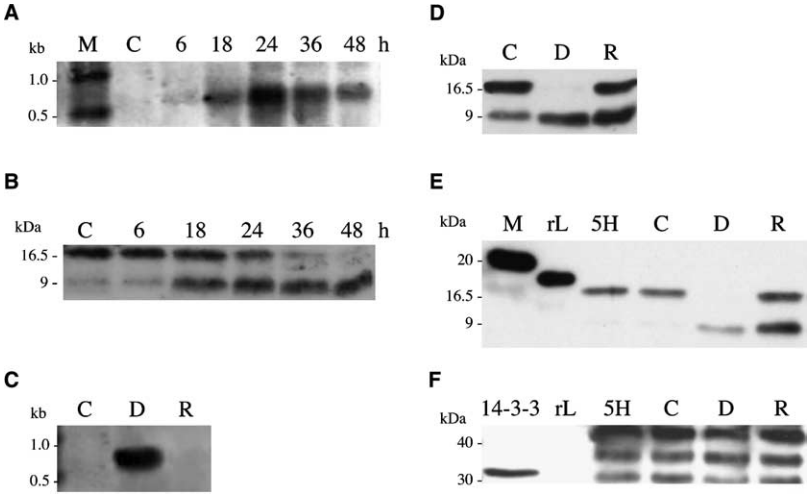


Fig. 2. Partially discordant expression of *Aav-lea-1* and its cognate protein. (A) Northern hybridisation showing expression of *Aav-lea-1* during 48 h exposure to 90% relative humidity; C: fully hydrated animals; M: 1 and 0.5 kb markers. (B) Western blot showing expression of AavLEA1 in the same nematode samples as A; protein size is indicated. (C) *Aav-lea-1* mRNA is lost on rehydration. C: hydrated nematodes; D: dried for 24 h at 90% RH; R: rehydrated overnight. (D) Western blot of AavLEA1 protein from same nematode samples as C. (E) Effect of hydration, dehydration and rehydration on AavLEA1 expression in *A. avenae*. M: 20 kDa marker; rL: recombinant AavLEA1 (18 kDa); 5H: nematodes soaked in water for 5 h; C: control animals, harvested and collected by sedimentation through water overnight (“wash harvest”); D: nematodes wash harvested and then dehydrated at 90% RH for 24 h; R: nematodes dehydrated, then rehydrated by soaking in water for 5 h. (F) Western blot with 14-3-3 antiserum of same extracts as E, except that a track containing protein extract from EGF-stimulated A431 cells (14-3-3) is included as a positive control.

this further, nematodes were harvested and subjected to air drying at 90% relative humidity (RH); these conditions are used to stimulate entry into anhydrobiosis and are known to induce expression of a range of genes associated with desiccation tolerance [15]. Nematodes were sampled at intervals over a 48 h period and both RNA and protein isolated. As anticipated, Northern hybridisation experiments showed that *Aav-lea-1* mRNA concentrations increased from a low level at the beginning of the experiment to a maximum at ~24 h after imposition of the stress, declining slightly thereafter (Fig. 2A).

Surprisingly, however, the corresponding LEA protein, whose predicted size is 16.5 kDa, was detected by Western analysis prior to dehydration, together with a smaller protein or group of proteins at around ~9 kDa. In fully hydrated animals, the full size protein predominated, but as the nematodes dehydrated the ratio of 16.5–9 kDa proteins shifted markedly until by the end of the experiment the antiserum detected mostly smaller protein(s) (Fig. 2B). When *A. avenae* was rehydrated overnight after 24 h at 90% RH, *Aav-lea-1* mRNA disappeared (Fig. 2C), whereas full length AavLEA1 protein again becomes detectable (Fig. 2D and E). Not all proteins behave in this manner on drying: e.g., three nematode 14-3-3 proteins detected by a monoclonal antibody showed no such dehydration-dependent shift in mass (Fig. 2F). Furthermore, stained SDS–PAGE gels of total protein extracts from dehydrated nematodes did not show any gross differences to those of hydrated animals (data not shown).

The presence of LEA protein in fully hydrated animals which contain little of its cognate mRNA is unusual but can probably be explained by differences in the stability of protein and mRNA. *A. avenae* is a fungivore grown in co-cultures of wheat grain and *Rhizoctonia solani*; during the later stages of culture, nematodes can swarm up the sides of the bottle after which they probably experience a degree of desiccation stress as the water film evaporates. Usually, nematodes are harvested by washing from the bottle with water (“wash harvest”) and sedimentation through a column of water; these animals contain very little *Aav-lea-1* mRNA (Fig. 3A). However, if swarming nematodes are recovered straight from the culture bottle, by scraping from the sides of the vessel (“scrape harvest”), they contain significant quantities of mRNA for the LEA protein. When these animals are soaked in water for as little as 10 min, the level of *Aav-lea-1* mRNA markedly decreases. As before, drying nematodes at 90% RH leads to elevation of mRNA levels. It seems therefore that expression of the *Aav-lea-1* gene and maintenance of mRNA levels are extremely sensitive to the hydration state of the nematode, and that some expression takes place in the culture, presumably reflecting a degree of water stress. Full size AavLEA1 is present in all harvested animals, and is not affected by length of time in water, whether 10 min, 5 h or 16 h, but again is lost in dried nematodes (Figs. 2E and 3B).

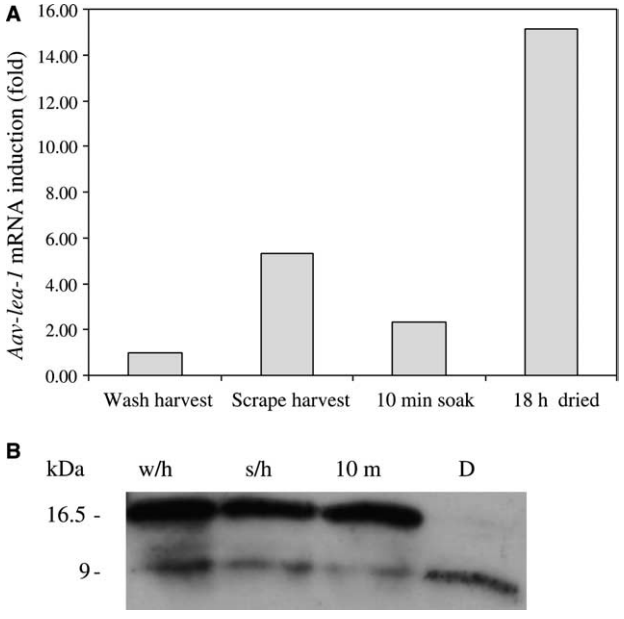


Fig. 3. Expression of *Aav-lea-1* and AavLEA1 under different harvest conditions. (A) Level of *Aav-lea-1* mRNA determined by quantitative PCR after conventional “wash harvest”, after harvesting by direct scraping from culture vessels (“scrape harvest”), or after an abbreviated wash harvest, where animals are exposed to water for only 10 min (“10 min soak”). Drying at 90% RH for 18 h after a wash harvest induces gene expression. (B) Western blot of protein extracted from the same nematode samples shows full-size AavLEA1 in all preparations, except when dried; w/h: wash harvest; s/h: scrape harvest; 10 m: abbreviated wash harvest; D: w/h animals dried for 18 h at 90% RH.

The presence of LEA protein in hydrated animals is presumably then due to *Aav-lea-1* gene expression in *A. avenae* prior to harvesting, but indicates that the half-life of the protein significantly exceeds that of its mRNA. The stability of full size AavLEA1 itself seems to be governed by dehydration since drying at 90% RH results in processing into smaller polypeptides. *Aav-lea-1* mRNA present in dried animals is likely to be stable in the dry state and, on rehydration, although the gene is switched off and stored mRNA begins to turn over, there is probably sufficient mRNA present for a burst of translation, with the resultant LEA protein once again showing a longer half-life than its message under these conditions.

### 3.3. LEA protein processing activity in extracts of dehydrating nematodes

The smaller forms of LEA protein in dehydrating nematodes suggest the presence of a processing activity which is induced by desiccation stress. To test this, we used protein extracts from both hydrated and dehydrated nematodes to treat recombinant AavLEA1 in vitro. Fig. 4A identifies an activity, presumably enzymatic, in protein extracts from dehydrating nematodes, which is able to process recombinant LEA protein into shorter forms resembling those seen in vivo. A much lower level of activity was seen in extracts from hydrated animals, suggesting that processing might be dehydration-specific; such an activity is novel, to our knowledge. The lack of processed LEA protein in control experiments indicates that spontaneous proteolysis is not occurring. Significantly, the processed recombinant LEA protein obtained by digestion with dehydrated nematode extract retained its ability to protect sensitive proteins from aggregation on drying. Fig. 4B shows the results of a light scattering assay which follows aggregate formation by mammalian citrate synthase (CS) on vacuum drying. Aggregation of CS is prevented by recombinant LEA protein, as reported previously [11], but processed material behaved similarly, demonstrating that the 9 kDa LEA protein products also have anti-aggregation activity.

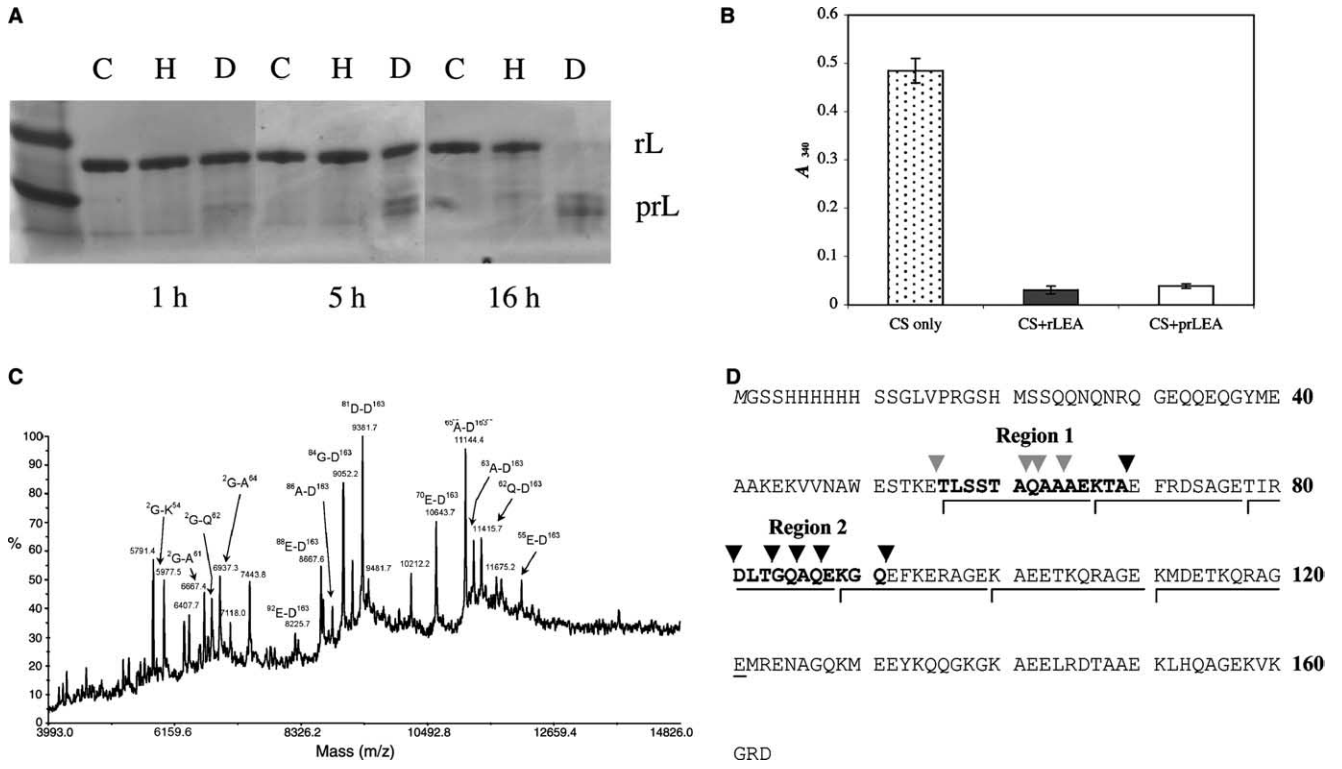


Fig. 4. LEA protein processing activity in dehydrating *A. avenae*. (A) Recombinant AavLEA1 was incubated with either water (C), protein extract from hydrated nematodes (H) or protein extract from dehydrated nematodes (D), respectively, in each of the three panels. Each panel represents incubation at ambient temperature (~20 °C) for 1, 5 or 16 h, respectively. (B) Light scattering assay showing apparent absorbance at 340 nm after vacuum drying of citrate synthase either alone (CS), with full-size AavLEA1 (CS + rLEA), or with processed AavLEA1 (CS + prLEA). (C) Linear MALDI-TOF spectrum showing signals for peptides derived from digestion of recombinant LEA protein with dehydrated nematode extract. Major cleavage positions were corroborated by nanoLC-MALDI-TOF/TOF tandem mass spectrometry after CNBr digestion (Supplementary Material). (**D**) Summary of major cleavage sites of recombinant LEA protein. Grey arrowheads indicate cleavage positions where both N- and C-terminal products are identified by mass spectrometry; black arrowheads show cleavage sites where only the C-terminal product has been observed. Clustered cleavage sites are indicated as Region 1 and Region 2, with sequence in bold. Group 3 LEA protein 11-mer motifs are underlined. The initiator Met (italicised) is not present in recombinant AavLEA1.

The processed LEA protein observed both in vivo and in vitro appears to be discrete in size, making it unlikely that it is due to degradation by non-specific proteases. Mass spectrometry was therefore performed to determine the position of cleavage points within recombinant AavLEA1. Analysis of the in vitro processed recombinant protein by MALDI-TOF-MS gave a mass spectrum with polypeptide signals which were tentatively assigned to the AavLEA1 sequence (Fig. 4C; Supplementary Material). These putative assignments were corroborated by cleavage of the in vitro processed recombinant protein samples with CNBr, which specifically hydrolyses polypeptides after methionine residues. The resulting peptides were identified by MALDI TOF/TOF tandem mass spectrometry (see Supplementary Material), which facilitated conclusive identification of most dehydration cleavage positions of AavLEA1. Similar results from replicate experiments confirm that cleavage is non-random with a preference for two regions, K54–A69 (region 1) and R80–Q91 (region 2), most frequently after R80; both regions are within tandemly arranged 11-mer motifs characteristic of Group 3 LEA proteins (Fig. 4D). Cleavage in region 1 can occur prior to that in region 2, since both cleavage products are otherwise intact, e.g., both polypeptides G2–A64 and A65–D163 are seen in Fig. 4C. In contrast, only the C-terminal products of cleavage in region 2 are observed, consistent with sequential cleavage events at region 1 and then at region 2.

Previously, we have proposed that LEA proteins exert their protein anti-aggregation function by behaving as "molecular shields" during water stress, sterically preventing interaction between partially unfolded proteins which would otherwise form toxic intracellular aggregates [11]. The data presented in this report develop this hypothesis further: the presence of AavLEA1 protein in nematodes under conditions where its gene is not expressed might allow a more rapid response to impending desiccation. Furthermore, processing AavLEA1 into smaller polypeptides could increase the specific activity of the LEA protein, whereby two shorter proteins are a more effective molecular shield than one larger one. Both expression pattern and processing of AavLEA1 could therefore act to increase both speed and magnitude of the protective activity of LEA protein in dehydrating nematodes.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2005.06.036.

## References


[1] Tunnacliffe, A. and Lapinski, J. (2003) Resurrecting Van Leeuwenhoek's rotifers: a reappraisal of the role of disaccharides in anhydrobiosis. Philos. T. Roy. Soc. B 358, 1755–1771.

[2] Cuming, A.C. (1999) LEA proteins in: Seed Proteins (Shewry, P.R. and Casey, R., Eds.), pp. 753–780, Kluwer Academic, Dordrecht, NL.

[3] Wolkers, W.F., McCready, S., Brandt, W.F., Lindsey, G.G. and Hoekstra, F.A. (2001) Isolation and characterization of a D-7 LEA protein that stabilizes glasses in vitro. Biochim. Biophys. Acta 1544, 196–206.

[4] Goyal, K., Tisi, L., Basran, A., Browne, J., Burnell, A., Zurdo, J. and Tunnacliffe, A. (2003) Transition from natively unfolded to folded state induced by desiccation in an anhydrobiotic nematode protein. J. Biol. Chem. 278, 12977–12984.

[5] Dure III, L. (2001) Occurrence of a repeating 11-mer amino acid sequence motif in diverse organisms. Protein Pept. Lett. 8, 115–122.

[6] Browne, J., Tunnacliffe, A. and Burnell, A. (2002) Plant desiccation gene found in a nematode. Nature 416, 38.

[7] Wise, M.J. and Tunnacliffe, A. (2004) POPP the question: What *do* LEA proteins do?. Trends Plant Sci. 9, 13–17.

[8] Sanchez-Ballesta, M.T., Rodrigo, M.J., Lafuente, M.T., Granell, A. and Zacarias, L. (2004) Dehydrin from *Citrus*, which confers in vitro dehydration and freezing protection activity, is constitutive and highly expressed in the flavedo of fruit but responsive to cold and water stress in leaves. J. Agric. Food Chem. 52, 1950–1957.

[9] Grelet, J., Benamar, A., Teyssier, E., Avelange-Macherel, M.-H., Grunwald, D. and Macherel, D. (2005) Identification in pea seed mitochondria of a late-embryogenesis abundant protein able to protect enzymes from drying. Plant Physiol. 137, 157–167.

[10] Reyes, J.L., Rodrigo, M.-J., Colmenero-Flores, J.M., Gil, J.-V., Garay-Arroyo, A., Campos, F., Salamini, F., Bartels, D. and Covarrubias, A.A. (2005) Hydrophilins from distant organisms can protect enzymatic activities from water limitation effects in vitro. Plant Cell Environ. 28, 709–718.

[11] Goyal, K., Walton, L.J. and Tunnacliffe, A. (2005) LEA proteins prevent protein aggregation due to water stress. Biochem. J. 388, 151–157.

[12] Sales, K., Brandt, W., Rumbak, E. and Lindsey, G. (2000) The LEA-like protein HSP12 in *Saccharomyces cerevisiae* has a plasma membrane location and protects membranes against desiccation and ethanol-induced stress. Biochim. Biophys. Acta 1463, 267–278.

[13] Koag, M.-C., Fenton, R.D., Wilkens, S. and Close, T.J. (2003) The binding of maize DHN1 to lipid vesicles. Gain of structure and lipid specificity. Plant Physiol. 131, 309–316.

[14] Hope, I.A., Ed., (1999). *C. elegans*. A Practical Approach, Oxford University Press, Oxford, UK.

[15] Browne, J.A., Dolan, K.M., Tyson, T., Goyal, K., Tunnacliffe, A. and Burnell, A.M. (2004) Dehydration-specific induction of hydrophilic protein genes in the anhydrobiotic nematode *Aphelenchus avenae*. Eukaryot. Cell 3, 966–975.

[16] Stephens, E., Sugars, J., Maslen, S.L., Williams, D.H., Packman, L.C. and Ellar, D.J. (2004) The N-linked oligosaccharides of aminopeptidase N from *Manduca sexta*. Site localization and identification of novel N-glycan structures. Eur. J. Biochem. 271, 4241–4258.

[17] Evans, A.A.F. (1998) Reproductive mechanisms in: The Physiology and Biochemistry of Free-Living and Plant-Parasitic Nematodes (Perry, R.N. and Wright, D.J., Eds.), pp. 133–154, CABI Publishing, Wallingford, UK.